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Differential Stability of PNS and CNS Nodal Complexes When Neuronal Neurofascin Is Lost

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Fast, saltatory conduction in myelinated nerves requires the clustering of voltage-gated sodium channels (Nav) at nodes of Ranvier in a nodal complex. The *Neurofascin* (*Nfasc*) gene encodes neuronal Neurofascin 186 (Nfasc186) at the node and glial Neurofascin 155 at the paranode, and these proteins play a key role in node assembly. However, their role in the maintenance and stability of the node is less well understood. Here we show that by inducible ablation of *Nfasc* in neurons in adult mice, Nfasc186 expression is reduced by >99% and 94% at PNS and CNS nodes, respectively. Gliomedin and NrCAM at PNS and brevican at CNS nodes are largely lost with neuronal neurofascin; however, Nav at nodes of Ranvier persist, albeit with ~40% reduction in expression levels. β IV Spectrin, ankyrin G, and, to a lesser extent, the β 1 subunit of the sodium channel, are less affected at the PNS node than in the CNS. Nevertheless, there is a 38% reduction in PNS conduction velocity. Loss of Nfasc186 provokes CNS paranodal disorganization, but this does not contribute to loss of Nav. These results show that Nav at PNS nodes are still maintained in a nodal complex when neuronal neurofascin is depleted, whereas the retention of nodal Nav in the CNS, despite more extensive dissolution of the complex, suggests a supportive role for the partially disrupted paranodal axoglial junction in selectively maintaining Nav at the CNS node.

Key words: neurofascin; nodes of Ranvier; sodium channels; myelination

Introduction

Myelinated axons conduct impulses rapidly because the α subunits of voltage-gated sodium channels (Nav) and their accessory proteins become clustered at nodes of Ranvier. In addition to voltage-gated sodium channels, the nodal complex includes β IV spectrin, ankyrin G, a neuronal isoform of neurofascin, Neurofascin 186 (Nfasc186), contactin (in the CNS), NrCAM, and gliomedin (in the PNS); Davis et al., 1996; Tait et al., 2000; Jenkins and Bennett, 2002; Yang et al., 2004; Eshed et al., 2005). The paranodal axoglial junctions flank the nodes in an adhesion complex comprising the glial isoform of neurofascin, Neurofascin 155 (Nfasc155), and the axonal proteins Caspr (also known as paranodin) and contactin (Menegoz et al., 1997; Tait et al., 2000;

Bhat et al., 2001; Boyle et al., 2001; Charles et al., 2002; Sherman et al., 2005).

The *Neurofascin* (*Nfasc*) gene is uniquely essential for the assembly of the nodal complex (Sherman et al., 2005). *Nfasc* encodes a neuronal isoform, Nfasc186, and a glial isoform, Nfasc155, by alternative splicing (Davis et al., 1996; Tait et al., 2000). Nfasc186 has a key role in clustering sodium channels (Zonta et al., 2008; Feinberg et al., 2010; Thaxton et al., 2011). The paranodal axoglial junctional complex can independently cluster Nav at nodes during development (Zonta et al., 2008; Feinberg et al., 2010), although a recent paper has disputed this (Thaxton et al., 2011).

Here, we show that there are significant differences between CNS and PNS nodal complexes in their susceptibility to disruption after the elimination of nodal neurofascin in the adult mouse. Nevertheless, in both cases, Nav are notably resistant to loss. We attribute this partly to the continued presence of intact paranodal junctions. However, age-related partial mislocalization of junctional proteins at CNS paranodes, exacerbated by the loss of nodal neurofascin, did not accelerate the decline in Nav maintenance at the node. We suggest that Nav are maintained at nodes through a combination of their interactions with nodal components in addition to neuronal neurofascin, together with restrictions on their diffusion caused by the paranodal adhesive junction, with the latter being particularly important in the CNS. This provides further evidence that the mechanisms of nodal stabilization depend on protein–protein interactions that are different from those that dominate initial assembly, as has been demonstrated for Nav at the axon initial segment (AIS; Zonta et al., 2011).

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Author contributions: A.D., B.Z., and P.J.B. designed research; A.D., B.Z., L.-M.N.W., and A.Z. performed research; A.D., B.Z., D.L.S., and P.J.B. analyzed data; D.L.S. contributed an essential reagent; P.J.B. and A.D. wrote the paper.

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*A.D. and B.Z. contributed equally to this work.

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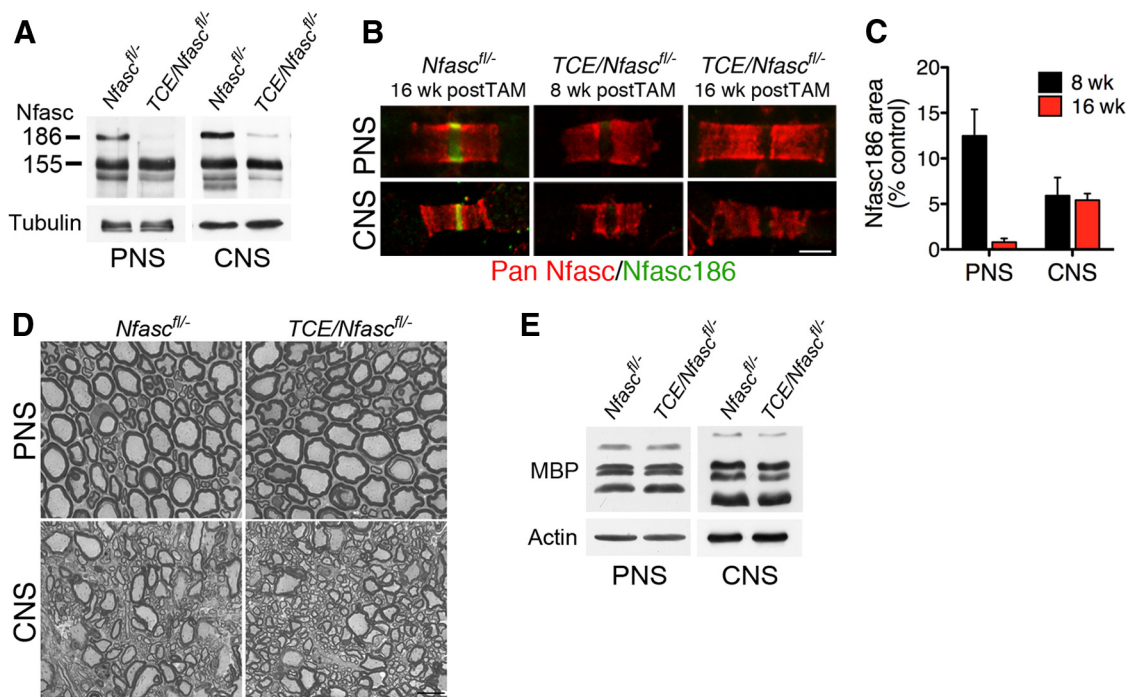


Figure 1. Characterization of Nfasc186 deletion and preservation of myelin in the *TCE/Nfasc^{fl/fl}* model. Control (*Nfasc^{fl/fl}*) and mutant (*TCE/Nfasc^{fl/fl}*) mice received intraperitoneal injections of tamoxifen to induce the recombination of the *Nfasc* floxed allele. **A**, Western blotting of peripheral nerve and spinal cord lysates from control and mutant mice 16 weeks post-tamoxifen shows substantial loss of Nfasc186, while glial Nfasc155 is unaffected. Tubulin was the loading control. **B**, Immunofluorescence of teased quadriceps nerves (PNS) and ventral funiculi of cervical spinal cord (CNS) from *Nfasc^{fl/fl}* and *TCE/Nfasc^{fl/fl}* mice shows the disappearance of nodal Nfasc186 at 8 weeks (wk) and 16 weeks post-tamoxifen in both PNS and CNS. The Pan Nfasc antibody recognizes both Nfasc186 and Nfasc155. Scale bar, 3 μ m. **C**, Quantitation of nodal area immunoreactive for Nfasc186 in mutant quadriceps nerves and ventral funiculi at 8 weeks and 16 weeks post-tamoxifen as a percentage of control. **D**, Toluidine blue-stained semithin transverse sections of quadriceps nerves (PNS) and ventral funiculi of cervical spinal cord (CNS) from *Nfasc^{fl/fl}* and *TCE/Nfasc^{fl/fl}* mice 16 weeks post-tamoxifen show no obvious alteration of myelin in myelinated axons. Scale bar, 10 μ m. **E**, Western blotting of peripheral nerve and spinal cord lysates 16 weeks post-tamoxifen shows normal amounts of the myelin basic proteins (MBP) in *Nfasc^{fl/fl}* and *TCE/Nfasc^{fl/fl}* mice. Actin was the loading control.

Materials and Methods

Animals. All animal work conformed to UK legislation (Scientific Procedures) Act 1986 and to the University of Edinburgh Ethical Review Committee policy, and mice were of either sex. Mice carrying alleles for *Nfasc^{-/-}*, *Nfasc^{fl/fl}*, and the characterization of *ThyCreERT2* (*TCE*) mice have been described (Sherman et al., 2005; Zonta et al., 2011). The *TCE* line was interbred with *Nfasc^{+/-}* and *Nfasc^{fl/fl}* mice to generate the *TCE/Nfasc^{fl/fl}* mice used for the present study. All mice were on the C57BL/6 background, and the quadriceps nerve and the ventral funiculus of the spinal cord were the source of PNS and CNS tissue, respectively. The tamoxifen injection protocol has been described previously (Zonta et al., 2011).

Antibodies, microscopy, and Western blots. Most primary and secondary antibodies, together with methods for immunofluorescence, electron microscopy, and Western blotting, have been previously described (Tait et al., 2000; Sherman et al., 2005; Zonta et al., 2008, 2011; Sherman et al., 2012). We also used rabbit anti-Gliomedin (1:500; E. Peles, Weizmann Institute of Science, Rehovot, Israel), mouse anti-Kv1.1, IgG2b K36/15 (1:100) (NeuroMab, UC Davis/NIH NeuroMab Facility), and rabbit anti-brevican (1:500; C. Seidenbecher, C.I. Leibniz Institute for Neurobiology, Magdeburg, Germany). Rabbit anti- β IV spectrin (1:100) and anti- β 1 sodium channel subunit (C terminus, 1:100) were generated after immunization with the TDEGNPKREGGERRASGRK and TSESKENCTGVQVAE synthetic peptides, respectively, each with an N-terminal cysteine conjugated to KLH. Each antibody was affinity-purified by immunoabsorption to a column of peptide coupled to sulfo-MBS-activated aminohexyl Sepharose 4B (Sigma). Samples were mounted in Vectashield (Vector Laboratories) and images were acquired with a confocal microscope (TCL-SL, Leica) using identical settings for control and mutant animals. For quantitation of stained nodal area, a series of four stacks per node of Ranvier was acquired under nonsaturating conditions. Nodal mean areas were obtained by averaging the values

of the four corresponding stacks. For all nodal area measurements, at least 30 nodes were analyzed per animal, with at least 3 animals per genotype.

Statistical analyses were performed by either Student's *t* test or one-way ANOVA followed by Tukey's multiple-comparison tests using GraphPad Prism 5.0c software. All figures were prepared using Adobe Photoshop CS4 extended version 11.

Nerve conduction velocity. Quadriceps nerves were transferred from oxygenated HEPES physiological solution (containing, in mM: 137 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5.5 D-glucose, and 5 HEPES, pH 7.2–7.4) to an isolated chamber containing an array of Ag/AgCl electrodes at 1 mm intervals, surrounded by liquid paraffin, and maintained at 37°C no longer than 10 min. Compound action potentials were recorded as described previously (Court et al., 2004). Recordings were repeated two to three times for each nerve. Conduction velocity was determined for each animal by averaging the mean conduction velocities from both nerves, and five animals were studied per genotype.

Results

Tamoxifen-induced loss of Nfasc186 at PNS and CNS nodes

Cre-mediated inactivation of the floxed allele in *TCE/Nfasc^{fl/fl}* mice was initiated by intraperitoneal injection of tamoxifen, at the end of which the mice were 6 weeks old. Loss of neuronal neurofascin was assessed by Western blot (Fig. 1A) and by immunofluorescence (Fig. 1B,C). Loss of Nfasc186 was confirmed by Western blot at 16 weeks and by immunofluorescence at 8 and 16 weeks post-tamoxifen (Fig. 1A,B). The isoform of myelin-forming glia, Nfasc155, was unaffected in both the CNS and PNS in the mutant (Fig. 1A). Quantitative immunofluorescence microscopy showed that loss of Nfasc186 at PNS nodes was essentially complete by 16 weeks post-tamoxifen (>99%), whereas

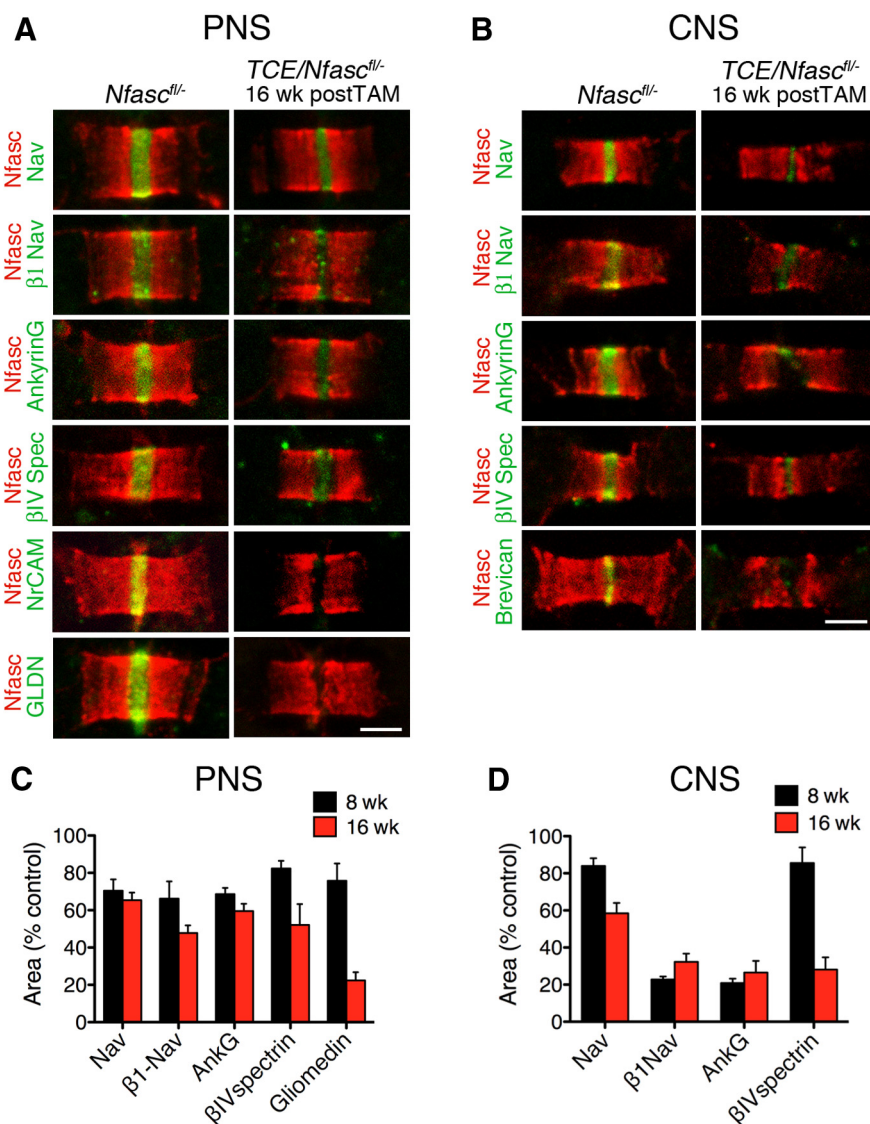


Figure 2. Loss of Nfasc186 leads to differential loss of nodal proteins in the PNS and CNS. **A, B**, Immunofluorescence of quadriceps nerves (PNS; **A**) and spinal cord ventral funiculi (CNS; **B**) from control (*Nfasc*^{+/+}) and mutant (*TCE/Nfasc*^{+/+}) mice at 16 weeks (wk) post-tamoxifen shows disruption of nodal components following inducible deletion of Nfasc186. β IV Spectrin; β IV Spectrin; GLDN, gliomedin. **C, D**, The quantitation of nodal disruption is shown for PNS (**C**) and CNS (**D**) where mean nodal areas \pm SEM for the mutant are expressed as percentage of control mean nodal area at 8 and 16 weeks post-tamoxifen treatment. Scale bar, 3 μ m.

~6% of CNS nodal Nfasc186 persisted. Interestingly, although ablation of neuronal neurofascin at PNS nodes was more complete than at CNS nodes, it seemed to proceed with different kinetics. At 8 weeks post-tamoxifen in the PNS, ~88% of the Nfasc186 was lost, whereas at the same time point in the CNS, nodal neurofascin levels had reached those observed at 16 weeks. Nevertheless, and as we have observed previously, the slow loss of nodal neurofascin by comparison with Nfasc186 at the AIS indicates that this protein is remarkably stable in the nodal complex (Zonta et al., 2011).

Loss of neuronal neurofascin did not affect the myelin sheath. Electron microscopy of transverse sections of PNS and CNS nerves from *Nfasc*^{+/+} and *TCE/Nfasc*^{+/+} mice 16 weeks post-tamoxifen did not reveal any obvious derangements to the sheath, and this was supported by Western blots of lysates from those nerves, which showed that myelin basic protein levels were normal in the mutants (Fig. 1 D,E).

Loss of neuronal neurofascin causes selective loss of nodal components

We assessed changes in immunofluorescence staining of nodal components by two methods: either we quantitated the area of staining under standardized conditions as described in Materials and Methods, or we measured the total pixel intensity of the immunostaining for individual proteins. We found that both methods gave essentially the same result. However, we found that pixel intensity measurements displayed much greater variance than the quantitation of area. Hence, all immunofluorescence quantitation is presented as area percentage of control.

NrCAM in the PNS and brevican in the CNS were completely absent from nodes at 8 weeks (data not shown) and 16 weeks post-tamoxifen (Fig. 2A,B). A third protein, gliomedin, which has also been proposed to interact directly with Nfasc186, was retained at high levels (~80% of control) up to 8 weeks but then declined to 20% of the control value by 16 weeks post-tamoxifen (Eshed et al., 2005; Fig. 2A,C). The delay in the loss of gliomedin may reflect its supplementary interactions with extracellular matrix at the PNS node (Eshed et al., 2007).

In contrast to gliomedin in the PNS, the amounts of $\beta 1$ subunit of the sodium channel ($\beta 1$ Nav), ankyrin G, and β IV spectrin were commensurate with those of Nav, and sodium channels themselves reached a level that did not change significantly from 8 to 16 weeks, namely between 65 and 70% of control values (Fig. 2A,C). These data suggested that a complex comprising Nav/ $\beta 1$ Nav/ankyrin G/ β IV spectrin remained clustered at the PNS node despite the almost complete absence of Nfasc186, although, of course, paranodes were still intact. Despite this limited resistance to disruption, the function of

quadriceps nerves was significantly compromised. Conduction velocities were reduced by 38% in *TCE/Nfasc*^{+/+} compared with *Nfasc*^{+/+} mice 16 weeks post-tamoxifen from 45.6 ± 3.5 to 28.1 ± 2.0 ms⁻¹ (mean \pm SEM; $p = 0.0024$; $n = 5$ per condition).

In the CNS we observed much more severe effects on the accessory protein $\beta 1$ Nav and the cytoskeletal linker proteins ankyrin G and β IV spectrin. When <6% of nodal Nfasc186 remained by 8 weeks post-tamoxifen, there were substantial reductions in the amounts of $\beta 1$ Nav and ankyrin G. In contrast, β IV spectrin and Nav were much less affected (>80% of control) (Fig. 2B,D). After another 8 weeks there were no further reductions in $\beta 1$ Nav and ankyrin G, but β IV spectrin at the node declined to levels that were now commensurate with these proteins (Fig. 2B,D). Surprisingly, Nav were less susceptible to disruption (Fig. 2B,D).

These data point to the differential effects of reducing neuronal neurofascin at the node of Ranvier in the CNS versus the PNS, which implies different mechanisms of stabilizing the nodal

complex at these two locations. A further difference in the consequences of withdrawing neuronal neurofascin from the node was the collateral effect on CNS paranodes, which was not observed in the PNS. The localization of glial neurofascin at the paranodes was extensively disrupted in the mutant by 16 weeks after tamoxifen treatment, although it can also be observed that staining in the CNS control was also not uniformly intact (Fig. 2B). To study this issue further in the CNS, we asked whether such apparent paranodal disruption might influence Nav retention at the node.

Given the more extensive disruption of the CNS compared with the PNS node, plus the significant reduction in PNS conduction velocity, it was perhaps not surprising that the mice died within 2 weeks of the last time point (16 weeks post-tamoxifen). This prevented us from determining whether further disruption of the PNS nodal complex followed the path of the CNS node.

Loss of neuronal neurofascin disrupts paranodes

Representative images of the distribution of Nav, Caspr, and Kv1.1 channels in control and mutant teased fibers from the spinal cord revealed both intact and disrupted paranodes (Fig. 3A). Disruption in the mutant was not a consequence of losing the transmembrane components of the junction since we had already shown that the amount of the glial Nfasc155 was unaffected in the mutant (Fig. 1A), and the other two neuronal components of this cell surface adhesion complex, Caspr and contactin, were similarly unaffected (Fig. 3B). To determine whether restriction of Nav to the node is influenced by the intactness of paranodal protein localization both in controls and when the levels of neuronal neurofascin are severely reduced, the mean Nav area was quantitated either when Kv channels were completely segregated to the juxtaparanodes (intact paranodes) or when Kv immunoreactivity invaded the paranode (disrupted paranodes).

Disrupted paranodes were observed in both control and mutant nerves (data not shown). Hence, we first determined whether control mice (*Nfasc*^{fl/-}) were significantly different from wild-type mice (*Nfasc*^{+/+}), and they were not (Fig. 3C). However, when the CNS nodal complex was deranged in mutant mice, there was an almost threefold increase in the number of disrupted paranodes compared with control mice (*Nfasc*^{fl/-}; Fig. 3C). Nevertheless, this deranged paranodal protein localization did not affect Nav retention in either control or mutant mice (Fig. 3D).

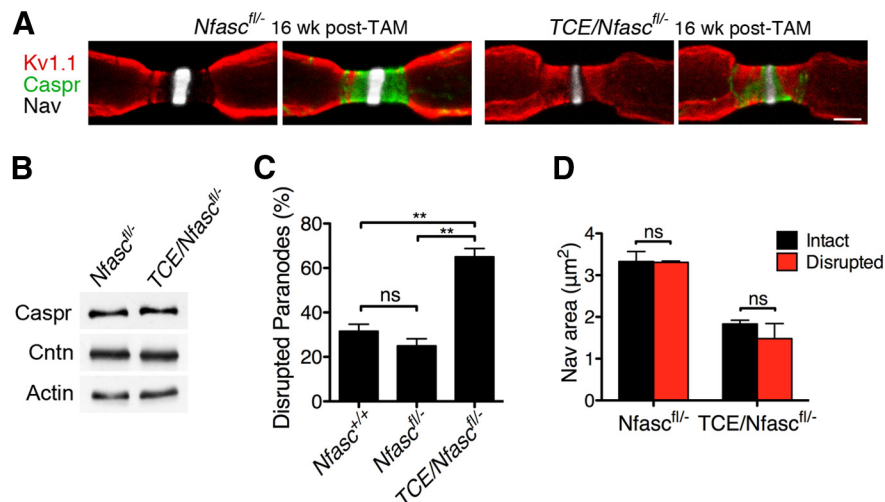


Figure 3. CNS paranodal and juxtaparanodal proteins are mislocalized following *Nfasc*186 deletion. **A**, Immunofluorescence of teased ventral funiculi from *Nfasc*^{fl/-} and *TCE/Nfasc*^{fl/-} mice 16 weeks (wk) post-tamoxifen treatment shows disrupted Caspr staining and relocation of voltage-gated potassium channel (Kv) at the paranodes of mutants. Scale bar, 3 μm. **B**, Western blotting of spinal cord lysates from control and mutant mice 16 weeks post-tamoxifen shows no reduction in Caspr or contactin in mutant nerves. Actin was the loading control. **C**, The percentage of paranodes displaying invasive Kv immunoreactivity was measured in wild-type (*Nfasc*^{+/+}), control (*Nfasc*^{fl/-}), and mutant (*TCE/Nfasc*^{fl/-}) mice 16 weeks post-tamoxifen (wild-type mice were the same age but not treated with tamoxifen) and are displayed as means ± SEM. There is a significant increase in disrupted paranodes in mutants compared with controls (one-way ANOVA followed by Tukey's multiple-comparison test; ***p* = 0.0001; *n* = 3, 120 paranodes per condition). ns, not significant. **D**, The mean areas of Nav immunoreactivity were measured when either paranodes were intact (Kv restricted to juxtaparanodes) or disrupted (Kv invasion of paranodes). In the control and the mutant, there is no significant difference (ns) in Nav localization at the nodes when paranodes are either intact or disrupted (mean ± SEM, one-way ANOVA followed by Tukey's multiple-comparison tests; *p* = 0.01, *n* = 3, minimum 90 nodes).

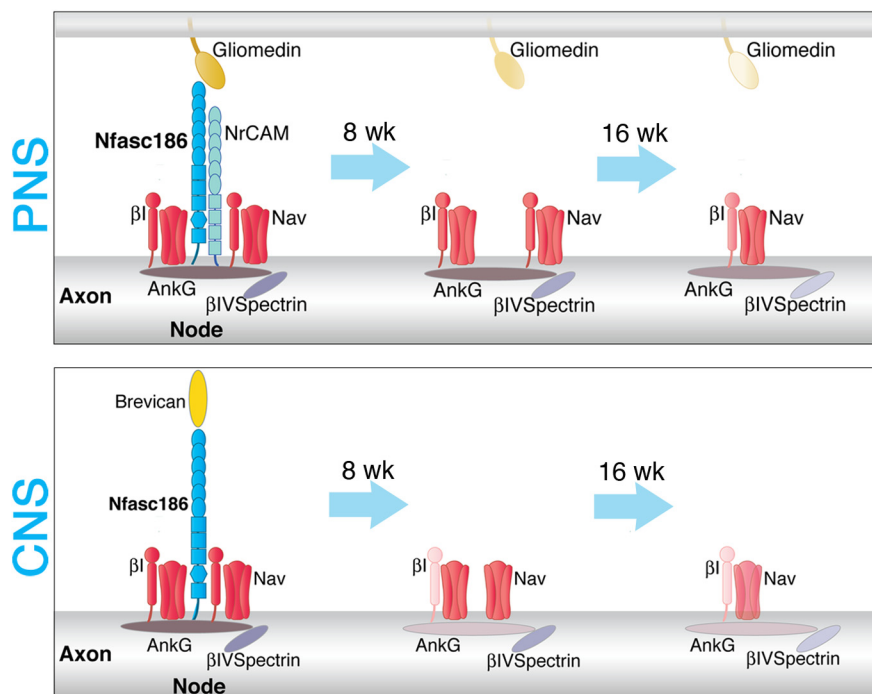


Figure 4. Diagram contrasting the sequence of events when *Nfasc*186 is lost in the PNS and CNS. The intensity of the colors indicates when proteins are diminished but not completely lost.

Discussion

The complete absence of NrCAM and brevican at PNS and CNS nodes, respectively, when depleted of *Nfasc*186 is consistent with considerable evidence for the direct interaction of *Nfasc*186 with these proteins (Hedstrom et al., 2008; Zonta et al., 2011). The loss

of 80% of gliomedin at PNS nodes was also consistent with previous observations on its interactions with Nfasc186 (Eshed et al., 2005), although residual gliomedin may reflect its ability to also interact with extracellular matrix proteins (Eshed et al., 2007). Similarly, although β 1Nav is known to interact with neuronal neurofascin (McEwen and Isom, 2004) in the PNS, this is not required to maintain it at the node, although reduced amounts would be predicted to affect the function of the channel, as we observed (Isom, 2002).

The fact that a complex comprising Nav/ β 1Nav/ankyrin G/ β IV spectrin remained largely clustered at the PNS node despite the almost complete absence of Nfasc186 suggested that intact paranodal axoglial junctions in the PNS restricted diffusion of this complex away from the node. Certainly, when paranodal axoglial junctions are completely disrupted, nodal components diffuse away (Rios et al., 2003; Pillai et al., 2009). In the PNS, where the nodal complex remained relatively intact, there was a substantial 38% reduction in the speed of nerve conduction. It is perhaps surprising that a 35% loss in Nav area should lead to such a significant reduction. However, this was accompanied by a 52% loss in β 1Nav, and this subunit is known to be an essential modulator of sodium channel function at nodes of Ranvier (Chen et al., 2004). Furthermore, the measured reductions of nodal components are based on area alone and may underestimate the overall loss in Nav, since there was also a reduction of pixel intensity per area (data not shown).

The more rapid loss of Nfasc186 after tamoxifen treatment in the CNS suggests that nodal Nfasc186 may be more stable at PNS nodes. Moreover, this greater loss in the CNS was accompanied by major reductions in the nodal content of β 1Nav and ankyrin G, even though Nav and β IV spectrin levels remained high (>80% of control). This suggests that in the CNS, interaction of Nfasc186 with β 1Nav and ankyrin G plays a more important role in stabilizing these proteins at the CNS node than in the PNS. Nevertheless, by 16 weeks post-tamoxifen, β IV spectrin in the mutant was reduced to levels commensurate with those of β 1Nav and ankyrin G, indicating that interaction of β IV spectrin with these former proteins, and most likely ankyrin G, is necessary to stabilize β IV spectrin at the node. Surprisingly, sodium channels were still retained to a large degree even at 16 weeks post-tamoxifen (~60% of control). Since brevicin was completely lost, it seems likely that paranodal axoglial junctions have a particularly important role in retaining Nav at the node, although the possible involvement of other β subunits of the sodium channel cannot be ruled out. Figure 4 is a diagram that summarizes the contrasting sequence of events in the PNS and CNS upon the loss of Nfasc186.

Age-related paranodal disruption has been reported in the CNS (Hinman et al., 2006). However, our data indicate that this does not exacerbate Nav loss at nodes lacking Nfasc186, at least in the time frame of these experiments. Nevertheless, it is of considerable interest that loss of neuronal neurofascin amplifies paranodal disruption. The precise mechanism by which this is achieved is not clear, but it seems likely to involve the axonal cytoskeleton since both ankyrin G and β IV spectrin are highly depleted at CNS nodes in the mutant. Disruption of paranodes due to loss of Nfasc186 during development has also been noted by others (Thaxton et al., 2011). In the latter study, disruption of paranodes was observed in both PNS and CNS. Similarly, in a companion article disruption of PNS paranodes was induced by the complete absence of gliomedin and NrCAM in peripheral nerves during development and adulthood (Amor et al., 2014). We only observed this phenomenon in the CNS, which may reflect the fact

we studied ablation in adult mice, where the relative stability of the preformed Nav/ β 1Nav/ankyrin G/ β IV spectrin complex at the PNS node retards or may even prevent further disturbance to the adjacent sub-paranodal axonal cytoskeleton altogether.

Given that many of the core components of the nodal complex are identical in the CNS and PNS, it was perhaps surprising that loss of neuronal neurofascin should differentially affect the stability of the nodal complexes in the PNS versus the CNS, and this difference was particularly pronounced for the cytoskeletal-associated protein ankyrin G, which is known to interact directly with Nfasc186 (Garver et al., 1997). Nevertheless, the fact that the retention of sodium channels in the CNS was comparable in the CNS and PNS does suggest that the axoglial junctions may have a particularly important function in maintaining Nav at the CNS node. It may also be the case that other ankyrin isoforms, known to be expressed in CNS neurons, compensate for the loss of ankyrin G (Kordeli and Bennett, 1991). This differential susceptibility to disruption may underpin the exacerbation of CNS axonal damage due to Nfasc186-specific antibodies in experimental allergic encephalomyelitis, an animal model of multiple sclerosis (Lindner et al., 2013).

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